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Point Mutation Causing Constitutive Signaling of CXCR2 Leads to Transforming Activity Similar to Kaposi’s Sarcoma Herpesvirus-G Protein-Coupled Receptor

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The chemokine receptor CXCR2 is the closest homologue to Kaposi’s sarcoma herpesvirus-G protein-coupled receptor (KSHV-GPCR), which is known to be constitutively activated and able to cause oncogenic transformation. Among G protein-coupled receptors, a DRY sequence in the second intracellular loop is highly conserved. However, the KSHV-GPCR shows a VRY sequence instead. In this study, we exchanged Asp$^{138}$ of the DRY sequence in the CXCR2 with a Val (D138V), the corresponding amino acid in KSHV-GPCR, or with a Gln (D138Q), and investigated the functional consequences of these mutations. In focus formation and soft agar growth assays in NIH 3T3 cells, the D138V mutant exhibited transforming potential similar to the KSHV-GPCR. Surprisingly, the CXCR2 wild type itself showed transforming activity, although not as potently, due to continuous autocrine stimulation, whereas the D138Q mutant formed no foci. In agreement with these results were high levels of inositol phosphate accumulation in the D138V mutant and the KSHV-GPCR, indicating constitutive activity. These data emphasize the importance of the DRY sequence for G protein-coupled signaling of the CXCR2. Either constitutive activation or persistent autocrine stimulation of the CXCR2 causes transformation similar to KSHV-GPCR-transfected cells, probably activating the same signal transduction cascade that can abrogate normal growth control mechanisms.

The human CXCR2 and Kaposi’s sarcoma herpesvirus-G protein-coupled receptor (KSHV-GPCR) are both members of the same rhodopsin/β-adrenergic subfamily of seven-transmembrane-spanning receptors. These receptors share characteristic structural features that presumably reflect their common mechanism of action (1).

The KSHV-GPCR is the product of open reading frame 74 of the human Herpesvirus 8. It has been speculated that the KSHV-GPCR has been pirated from CXCR2 or CXCR1 (2), its closest homologues.

The KSHV-GPCR exhibits constitutive signaling via activation of phosphoinositide-specific phospholipase (3) in the absence of ligand, although it can bind IL-8 and other chemokines. The CXCR2 activates the same signal transduction cascade, when it binds its ligands that include IL-8, GRO-α, and NAP-2, but does not exhibit constitutive signaling.

The KSHV-GPCR appears to be involved in the pathogenesis of Kaposi’s sarcoma (4) and primary effusion lymphomas (5). Expression of this receptor stimulates proliferation in rat fibroblasts (3) and causes transformation in NIH 3T3 cells and tumors, when transfected cells are injected into nude mice (6). This transforming activity of the receptor is thought to be a result of its constitutive activation.

The DRY sequence at the junction of the third transmembrane domain to the second intracellular loop of the CXCR2 is a highly conserved motif among G protein-coupled receptors. However, the KSHV-GPCR shows a VRY motif in this position.

It has been shown for otherGPCRs, e.g., the α$^{1b}$-adrenergic (7, 8), the β$^{1}$-adrenergic receptor (9), and the AT$^{1A}$ angiotensin II receptor (10), that mutations in the second and third intracellular loop result in increased agonist-independent receptor activity and confer sometimes oncogenic properties to the receptor (11). In addition, mutations inducing constitutive activation of GPCRs have been described and found to be associated with human diseases (12, 13).

This prompted us to compare the activity and function of the KSHV-GPCR and the CXCR2, and to introduce mutations into the highly conserved DRY sequence of CXCR2.

In this study, we report that the replacement of Asp$^{138}$ by Val in the second intracellular loop of the CXCR2 constitutively activates the receptor and causes transformation in transfected NIH 3T3 cells comparable with results seen with the KSHV-GPCR. Furthermore, we show for the first time that the CXCR2 wild type itself is able to transform transfected NIH 3T3 cells due to autocrine stimulation by mouse KC, the mouse equivalent of GRO-α, which binds to the CXCR2 and is produced by mouse fibroblast NIH 3T3 cells. These data provide support for a role of the CXCR2 in cell proliferation and tumorigenesis.

Materials and Methods

DNA constructs, transfection, and cell culture

DNA constructs. The CXCR1 and CXCR2 receptor constructs have been described (14). The KSHV-GPCR was amplified by RT-PCR from BCB-1 lymphoma cell RNA (obtained from Dr. Jaques Corbeil, University of California, San Diego) using the 5’ oligo GAGAATTCAGGCCAT...
were counted after 2 wk.

In addition, 200 stably transfected cells were seeded on a layer of DNA per 60-mm dish in quadruplicates, and cell foci were counted after 3 wk.

A total of 4 NIH 3T3 cells were transfected with the receptor constructs (1 μg DNA per well) in quadruplicates, and cell foci were counted after 10 min at room temperature, followed by 20 min at 37°C. Inositol phosphates were extracted with 10% perchloric acid, followed by neutralization with KOH/Tris. 3H]Inositol phosphates were separated by anion-exchange chromatography on AG 1-X8 formate resin (Bio-Rad, Vista, CA), as described by Berridge (19). Inositol phosphate accumulation was expressed as the percentage of 3H label in inositol phosphates per μg of protein in the cell precipitates.

Pertussis toxin (100 ng/ml) was added for 16 h before inositol phosphate extraction. Anti-CXCR2 and anti-mouse KC Abs (R&D Systems) were added together with the [3H]myo-inositol for 48 h.

Ca2+ mobilization and actin polymerization

RBL2H3 cells stably transfected with the different receptor constructs were labeled with Indo-1AM (Molecular Probes, Eugene, OR), and Ca2+ flux was measured on an SLM 8000 fluorometer (Spectronic Instruments, Rochester, NY), as previously described (14). Actin polymerization in RBL2H3 cells was measured as described earlier (20). All results are plotted relative to the mean fluorescence of the sample before addition of the chemokine.

Results

Focus formation and growth in soft agar

The consequences of expression of CXCR2, CXCR1, mutant receptors, and KSHV-GPCR on cell growth were investigated in NIH 3T3 cells. Focus formation in these cells represents a morphologic manifestation of transformation associated with the loss of contact inhibition that limits the cell density of these cells.

The focus formation assay was performed in two different ways by plating 200 stably transfected NIH 3T3 cells on a layer of untransfected cells or by transient transfection (Figs. 2 and 3). Focus formation by cells expressing the KSHV-GPCR served as the positive control. As expected, the KSHV-GPCR caused high numbers of focus formation, which confirms the results described earlier (6).

Surprisingly, cells transfected with the wild-type CXCR2 also formed foci, although to a lesser extent, which were observed with both methods and not seen with untransfected NIH 3T3 fibroblasts, those transfected with the pSFFV.neo vector only, or with the CXCR1 (Figs. 2 and 3). Cells within foci caused by the CXCR2 manifested the malignant phenotype with a loss of density-dependent growth inhibition, resulting in increased cellular packing and piling up of cells. This oncogene-like ability of the CXCR2 to induce transformation is presumably due to continuous autocrine stimulation of the receptor by mouse KC, the mouse equivalent of GRO-α, produced by NIH 3T3 cells. Overnight cultures of NIH 3T3 cells contained between 12–20 ng/ml of mouse KC, which is a sufficient concentration to activate the human CXCR2 (21).

The mutant receptor D138V, in which the Asp of the DRY motif is exchanged for the corresponding hydrophilic Gln, produced as many foci as the KSHV-GPCR. In contrast, the CXCR2 mutant D138Q, in which the Asp was replaced by the hydrophilic Gln, induced a complete loss of focus formation by cells transfected with the wild-type CXCR2 also formed foci, although to a lesser extent, which were observed with both methods and not seen with untransfected NIH 3T3 fibroblasts, those transfected with the pSFFV.neo vector only, or with the CXCR1 (Figs. 2 and 3). Cells within foci caused by the CXCR2 manifested the malignant phenotype with a loss of density-dependent growth inhibition, resulting in increased cellular packing and piling up of cells. This oncogene-like ability of the CXCR2 to induce transformation is presumably due to continuous autocrine stimulation of the receptor by mouse KC, the mouse equivalent of GRO-α, produced by NIH 3T3 cells. Overnight cultures of NIH 3T3 cells contained between 12–20 ng/ml of mouse KC, which is a sufficient concentration to activate the human CXCR2 (21).

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FACS analysis indicated that surface expression of the CXCR1, CXCR2, and the D138V and D138Q mutants was similar, even a little higher for the D138Q mutant (data not shown), so that differences in the level of expression could not explain the varying behavior of these receptors.

The transforming potential of the CXCR2, mutant receptors, and the KSHV-GPCR was paralleled by their ability to form colonies in soft agar. Cells transfected with the CXCR2, the D138V mutant, or the KSHV-GPCR all formed colonies after 2–3 wk in culture (Fig. 4). Colonies of CXCR2-transfected cells tended to stay smaller than those of cells transfected with the KSHV-GPCR or the D138V mutant, but clearly grew in an anchorage-independent fashion, usually associated with the potential to metastasize (22). Untransfected cells or those transfected with the D138Q mutant or the CXCR1 failed to grow in soft agar (Fig. 4).

**Inositol phosphate accumulation**

Activation of phospholipase C is a major pathway that links G protein-coupled receptor activation to cell proliferation (3, 23). To evaluate G protein-coupled signaling through this pathway, we measured inositol phosphate accumulation in NIH 3T3 cells stably transfected with the various receptors and the mutants.

In agreement with previous reports (3), NIH 3T3 cells transfected with the KSHV-GPCR accumulated 3.5 times the amount of inositol phosphates observed in untransfected NIH 3T3 cells. This increase could not be blocked by pertussis toxin treatment of these cells, which only caused small, nonspecific inhibition of inositol phosphate accumulation that was seen in all cells treated with pertussis toxin (Fig. 5). Pertussis toxin inhibited inositol phosphate accumulation (Fig. 5). However, stimulation of this pathway in CXCR2-transfected cells seemed to be mediated by mouse KC, since either Abs to mouse KC or to the CXCR2 inhibited inositol phosphate accumulation (Fig. 5). Pertussis toxin, which inactivates G, largely, but not completely, blocked inositolphosphate formation (Fig. 5).

In contrast, accumulation of inositol phosphates in cells transfected with the CXCR2 mutant D138V (3.1-fold higher than found in untransfected NIH 3T3 cells) could not be blocked by either of...
the Abs, and pertussis toxin blocked inositol phosphate accumulation in these cells only to a small degree (Fig. 5). Together these results suggest that this mutant receptor signals in an agonist-independent fashion similar to the KSHV-GPCR.

The CXCR2 mutant D138Q, in which the same Asp was replaced with Gln, showed only a small increase of inositol phosphate turnover over untransfected NIH 3T3 cells (Fig. 5), which is consistent with the low potential of this mutant to form foci. A similar behavior was seen in cells transfected with the CXCR1.

Ca$^{2+}$ mobilization and actin polymerization in RBL2H3 cells

The same receptor constructs were transfected into RBL2H3 cells, and calcium mobilization and actin polymerization were determined.

As shown in Figs. 6 and 7, CXCR2- and CXCR1-transfected cells showed a high level of actin polymerization and calcium mobilization upon stimulation with IL-8. At high concentrations, the CXCR2 mutant D138Q responded almost as well as the CXCR2, but the mutant showed a much lower affinity. Approximately 20-fold higher concentrations of ligand were necessary for a response equal to that seen with the wild-type receptor. These functional results were confirmed by $^{125}$I-labeled IL-8 binding studies that showed a $K_d$ of $1.2 \times 10^{-9}$ M for the CXCR2 and a $K_d$ greater than $2 \times 10^{-8}$ M for the D138Q mutant. The affinity of GPCRs not coupled to G proteins generally drops by 1–2 orders of magnitude. All of these results are consistent with poor G protein coupling of the D138Q mutant.

In CXCR2-transfected cells, the Ca$^{2+}$ mobilization was transient and Ca$^{2+}$ concentrations quickly fell back to almost background levels, whereas Ca$^{2+}$ remained elevated over a prolonged time in D138Q-transfected cells. Ca$^{2+}$ mobilization and actin polymerization could be completely blocked by pertussis toxin in CXCR1, CXCR2, and D138Q (data not shown).

In both assays, the KSHV-GPCR and the mutant D138V showed only a small response to IL-8 stimulation, consistent with the idea that these receptors exhibit constitutive activity independent of ligand. In accordance with the report of Gershengorn et al. (2), the KSHV-GPCR as well as D138V can be activated by IL-8 over constitutive levels, which could mean that additional stimulation might even enhance the functional activities of these receptors.

Discussion

Our studies demonstrate the potential of the CXCR2 and its ligands to activate signal transduction pathways that can abrogate normal growth control mechanisms.

It has been demonstrated for several receptors that discrete mutation can cause dramatic increases in agonist-independent receptor activity (24). It was therefore proposed that important conformational constraints maintain the receptor preferentially in an...
inactive conformation and that these constraints are released upon activation, causing key sequences to be exposed to the G protein (25). Single point mutations seem to be able to change the receptor conformation leading to constitutive activation, thereby mimicking the active state of the wild-type receptor.

Constitutive signaling, as shown by some native GPCRs (26), occurs more commonly with mutated GPCRs (24, 27) in several human diseases and in tumors (28, 29). The KSHV-GPCR has been recently described as a naturally occurring receptor exhibiting constitutive signaling, which is thought to play a role in the pathogenesis of Kaposi’s sarcoma. It has been speculated that the KSHV pirated the gene encoding for the KSHV-GPCR from CXCR2 or CXCR1, its closest homologues, and retaining similarities to these receptors.

Our study points out the importance of the DRY sequence in CXCR2 for regulated signaling function and the significance of the mutation to a VRY motif, the respective sequence in the KSHV-GPCR, which makes the CXCR2 functionally similar to the KSHV-GPCR. The DRY sequence at the junction between the third transmembrane domain and the second intracellular loop is shared by almost all GPCRs, including the chemokine receptors. The importance of this sequence for G protein coupling has been shown for the CCR5 (30). The surrounding amino acids are also highly conserved among chemokine receptors and necessary for proper signal transduction (31). Sequence homology between the CXCRs and the KSHV-GPCR is higher in this region than in any other area outside of the transmembrane domains.

The second intracellular loops of both the CXCR2 and the KSHV-GPCR contain the basic amino acids at both the NH₂- and COOH-terminal ends that are necessary for interaction with G proteins (32, 33).

Replacement of the Asp in the DRY sequence by the bulky hydrophobic Val in this location, as seen in the KSHV-GPCR and in our D138V mutant, presumably pulls this region into the plasma membrane. The more conservative D138Q mutant would not be expected to have this effect. It has been proposed that the α-helical structure of the third transmembrane domain of GPCRs continues on into the second intracellular loop. Hydrophobic residues of the second intracellular loop are thought to interact with α-helical structures of the third intracellular loop, while amino acids in the second intracellular loop that face away from the third intracellular loop are considered important to keep the receptor in an active state in the absence of ligand (34). Our results are consistent with this hypothesis.

In contrast, continuous stimulation of the CXCR1 with human IL-8 or introducing the point mutation that exchanges the Asp for a Val into the CXCR1 receptor (D134V-CXCR1) did not result in transforming capacity (Fig. 2), although both the CXCR1 and the mutant receptor were expressed as assessed by FACS (data not shown). Hence, the similarity in the signaling of the CXCR2 mutant D138V and the KSHV-GPCR supports the hypothesis that the gene of the KSHV-GPCR has been pirated from the CXCR2. In addition, it emphasizes the transforming potential of the CXCR2, which seems to be exhibited only by the CXCR2 and not by the CXCR1. Being continuously stimulated or constitutively activated due to a single amino acid change in the receptor, the CXCR2 seems to activate normal cellular genes with latent transforming potential and subvert regulatory key pathways controlling cell proliferation. There have not been any reports about the transforming potential of the CXCR2 to date, although Norgauer et al. (35) showed that the CXCR2 exhibits a growth-promoting function in melanoma cells that could be blocked by Abs against the CXCR2.

It also has been shown for other G protein-coupled receptors that persistent activation can transform cells (18) and act as onco-

genes in human cancers (29). These pathways are complex (23) and not understood in detail. Increased inositol phosphate turnover seems to play a role, and indeed occurred in all focus-forming mutants we analyzed.

Leukocytes and leukocytic cell lines such as RBL2H3 cells express such a high level of Gαq, that the signal transduction cascade in these cells following stimulation with IL-8 is dominated by the activation of Gαq (36). The CXCR2 can, however, also couple to Gα14 and Gα16 (37), and possibly additional G proteins. Pertussis toxin largely, but not completely, blocked inositol phosphate accumulation in NIH 3T3 cells expressing the CXCR2, while it abrogated functions induced by IL-8 in RBL2H3 cells expressing the same receptor. Indeed, actin polymerization, which was blocked completely by incubation with pertussis toxin in RBL2H3 cells, was not affected by this G and G protein-specific inhibitor in NIH 3T3 cells (Schräufstatter et al., unpublished observation). Another G protein, possibly Gα12 or Gα13, must mediate this response in NIH 3T3 cells. Gα12 has recently been described to be involved in cellular transformation caused by m1 muscarinic acetylcholine receptors in NIH 3T3 cells (38). The relative contribution of various G proteins in CXCR2-transfected NIH 3T3 cells warrants further evaluation.

Expression of the CXCR2 has been shown to be present on many different cell types, including leukocytes and related cell lines, melanoma cells, and breast cancer cells (39). IL-8 and Gro-α are produced by various cell types and are known to be angiogenic and mitogenic for endothelial cells (40). But the IL-8 mitogenic signaling pathway has not been defined, and cellular chemokine receptors have not been tested carefully for mitogenic activity. In addition, it has been proposed repeatedly that IL-8 acts as an autocrine promoter of cell proliferation (41) and even human tumor growth, e.g., in non-small cell lung cancer (42) or gastric carcinoma (43).

These reports together with our findings suggest that IL-8 produced by tumor cells may regulate cell proliferation and tumor growth in an autocrine fashion via CXCR2. The KSHV-GPCR in Kaposi’s sarcoma might thus be used to connect constitutively to the pathway that is used for IL-8–induced angiogenesis, one of the main features in Kaposi’s sarcoma histology besides inflammation and proliferation.

In conclusion, we propose that IL-8 and other ligands of CXCR2 may act as autocrine growth factors in tumors or at sites of inflammation through the activation of CXCR2, and that the KSHV-GPCR may use the same signal transduction cascade as the CXCR2.

References


